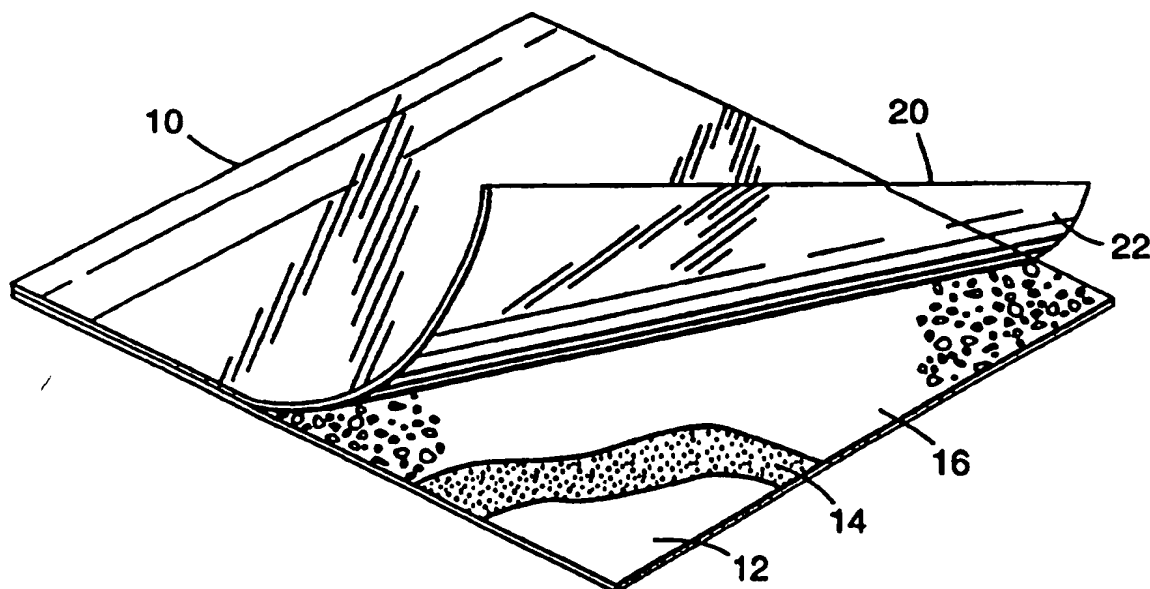




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(54) Title: THIN FILM CULTURE PLATE DEVICE CONTAINING GRANULATED MEDIUM PARTICLES

**(57) Abstract**

The present disclosure reports a thin film culture plate device including i) a self-supporting, waterproof substrate containing a layer of a unique reconstitutable culture medium made of nutrients for growing microorganisms, and a mixture of gelling agents which are prepared in granular form by agglomerating the nutrients and mixture of gelling agents in the presence of an aqueous binder and ii) a cover sheet adhered to a portion of the substrate. Methods to make agglomerated medium particles are also reported.

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THIN FILM CULTURE PLATE DEVICE CONTAINING GRANULATED MEDIUM PARTICLES

This invention is generally related to thin film culture plate devices which are
5 used to detect and enumerate bacteria which are present in a sample and particularly
related to a thin film culture plate device which contains granulated medium
particles made of nutrients and a mixture of gelling agents.

BACKGROUND

10 Classical methods for determining the presence and number of bacteria in a
sample are time consuming, tedious and labor intensive. Typically, a technician must
prepare reagents and nutrients, mix the nutrients with agar, heat the mixture, pour the
mixture into a petri dish, allow the agar to gel, obtain a test sample, dilute the test
15 sample, add an aliquot of the diluted sample to the agar, incubate the inoculated plate for
24-48 hours and finally count the number of growing bacterial colonies in the petri dish.
Products and processes which reduce the preparation time and which facilitate a count
of the bacterial colonies would clearly be welcomed by those working in this field.

One example of a product which greatly simplifies the above preparation time is
a thin film culture plate device for growing microorganisms that is described in U.S.
20 Patent 4,565,783 as well as variations of this device such as those described in U.S.
Patent Nos. 5,089,413; 5,137,812 and 5,232,838. In a typical thin film culture plate
device, a reconstitutable dry powder containing a gelling agent and microbial growth
nutrients is coated on a waterproof substrate. A transparent, read-through cover sheet
coated on a surface with an acrylate adhesive containing an indicator dye and powdered
25 gelling agent is attached to the coated substrate.

When the above device is used, a predetermined amount of an aqueous sample
is typically placed in contact with the coated substrate and the cover sheet is placed over
the sample and substrate. The aqueous sample hydrates the soluble dry powder which
then forms a gelled medium capable of sustaining microbial growth. During the growth

period, the indicator dye adhered to the cover sheet reacts in the presence of viable microorganisms to give a detectable response that allows visualization of bacterial colonies which are grown on the culture device. Thin film culture plate devices are commercially available and are sold under the tradename PETRIFILM plates by 3M,
5 St. Paul, MN.

Thin film culture plate devices are generally much simpler to use than conventional agar medium/petri dish systems because there is no need for the user to heat and mix the growth medium, agar and other reagents and then add the mixture to petri dishes or pour plates. In addition, these devices are compact and easily disposed
10 of and therefore are easier and safer to use.

In spite of the many advantages that thin film culture plate devices have over conventional types of culture systems, the utility of these thin film plates for certain applications may be challenged under certain conditions, microbes and samples. For example, the limited amount of gelling agent in the device may be insufficient to remain
15 in a semi-solid state when inoculated with certain samples containing some microorganisms. Briefly, some samples which contain certain bacteria commonly known as "liquifiers" will cause the growing colonies to overrun the semi-solid gel and thus hinder detection and enumeration of such microorganisms.

The present invention addresses the difficulties presented in attempting to
20 grow, detect and enumerate a wide variety of microorganisms using thin film culture plate devices.

SUMMARY OF THE INVENTION

This invention provides a thin film culture plate device containing novel
25 medium particles. The thin film culture plate devices of this invention have an expanded range of use for detecting and enumerating bacteria in bacteria-containing samples. In part, this invention is directed to a thin film culture plate device including i) a self-supporting, waterproof substrate containing a layer of a unique reconstitutable culture medium of nutrients for growing microorganisms and a
30 mixture of gelling agents which are prepared in granular form by agglomerating the

nutrients and mixture of gelling agents in the presence of an aqueous binder in a fluidized bed and ii) a cover sheet adhered to a portion of the substrate.

In one embodiment of this invention, the thin film culture plate device includes a substrate made of a film or sheet of polyester and a cover sheet made of a film or sheet of transparent polyethylene. In this embodiment, an agglomerated, particulate, reconstitutable culture medium or medium particles are adhered to the substrate and to the cover sheet with a nontoxic adhesive.

In yet another embodiment, the thin film culture plate device may optionally include a hydrophobic spacer adhered to a surface of the substrate which is adapted to retain an amount of a sample or a liquid in contact with the reconstitutable culture medium.

This invention also includes, in part, a method of making medium particles which includes the step of agglomerating nutrients for growing microorganisms and a mixture of gelling agents in the presence of an aqueous binder in a fluidized bed.

In a preferred embodiment of this invention, microbial nutrients such as proteins, carbohydrates and salts are combined with gelling agents such as xanthan, locust bean, and guar gums or mixtures thereof. A preferred mixture of these materials includes about 15-25 wt.% nutrients and about 75-85 wt.% gelling agents. This mixture of the nutrients and gelling agents is initially contacted with a sufficient amount of an aqueous binder to form particles having particle diameters greater than about 40 microns and then further drying of the particles at a temperature less than about 40°C provides medium particles having a moisture content of less than about 5 wt.%. Preferred medium particles prepared according to this method generally have particle diameters in the range of about 50-150 microns and particle densities in the range of about 0.5-0.6 g/cm³.

Additional advantages and features which further characterize and describe the present invention are reported in the accompanying drawing and detailed description and are recited in the appended claims.

cross-linking which allows the medium to be exposed to gamma radiation, because if the media is not cross-linked the gamma radiation will break down the gum.

The output specifications of the media particles include that at least 70% of the particles be in the range of 325 to 140 mesh, and that the moisture be less than
5 5%.

The medium particles may be applied by any means suitable for the application of a substantially uniform layer. Preferred methods include the use of a shaker-type device, or the use of a powder coater.

Fig. 1 illustrates a thin film culture plate device suitable for use with the medium
10 particles of the present invention. Briefly, these types of devices are described in U.S. Patent 4,565,783 and U.S. Patent 5,089,413 both of which describe processes of making and using these types of thin film culture plate devices.

The thin film culture plate device 10 includes a body member having a self-supporting, waterproof substrate 12. Substrate 12 is preferably a relatively stiff
15 material made of a waterproof material that does not absorb water such as polyester, polypropylene, or polystyrene. For example, polyester films approximately 100 μ to 180 μ thick, polypropylene films approximately 100 μ to 200 μ thick, and polystyrene films approximately 300 μ to 380 μ thick have been found to work well with the present invention. Other suitable substrates include paper with a polyethylene or
20 other substantially water-proof coating, such as "Schoeller Type MIL" photoprint paper (Schoeller, Inc., Pulaski, NY). In addition, substrate 12 can be transparent, translucent, or opaque, depending on whether one wishes to view and count microorganism colonies through substrate 12.

The upper surface of substrate 12 is coated with a layer of adhesive 14 and then
25 further coated with reconstitutable culture medium particles 16 of this invention. The adhesive should be sufficiently transparent when hydrated to allow viewing of bacterial colonies growing on the surface of the substrate through the coated substrate.

The adhesive should also be coated on the substrate in a thickness which allows the substrate to be uniformly coated with medium particles without completely
30 embedding the medium particles in the adhesive. Suitable adhesives are coated (preferably, knife-coated) onto substrate 12 at a thickness that is preferably less

than the diameter of the medium particles 16 adhered to adhesive layer 14. Typically, enough adhesive composition is used to facilitate adherence of the medium particles to upper surface 14 of substrate 12, but not so much that the particles become completely embedded in the layer of adhesive. Generally, an
5 adhesive coating weight of about 0.15 g/24 square inches or higher (dry coating weight) is suitable.

The adhesive composition preferably is a pressure-sensitive adhesive. More preferably, the adhesive is a pressure-sensitive adhesive such as a water-insoluble adhesive comprising a copolymer of an alkyl acrylate monomer and an alkyl amide
10 monomer. Preferably the weight ratio of alkyl acrylate monomer to alkyl amide monomer in these copolymers is from about 90:10 to 99:1, more preferably 94:6 to 98:2. The alkyl acrylate monomer may be a lower alkyl (C_2 to C_{10}) ester monomer of acrylic acid, including, without limitation, isooctyl acrylate (IOA), 2-ethylhexyl acrylate, butyl acrylate, ethyl acrylate, isoamyl acrylate, and mixtures of these
15 monomers, while the alkyl amide monomer may be, without limitation, acrylamide (ACM), methacrylamide, N-vinylpyrrolidone (NVP), N-vinylcaprolactam (NVCL), N-vinyl-2-piperidine, N-(mono- or di-lower alkyl (C_2 to C_5))(meth)acrylamides, N-methyl(meth)acrylamide, N,N-dimethyl(meth)acrylamides, or mixtures these monomers. Suitable water-insoluble adhesive copolymers include a copolymer of
20 IOA and ACM, or an aqueous emulsion suspension of a copolymer of IOA and NVP, as described in U.S. Patent No. 5,232,838. Alternative adhesives may include an adhesive formed by aqueous emulsion polymerization as described in U.S. Patent 5,424,122.

Appropriate adjustments to the pH of the adhesive are made, as needed, to
25 insure that the adhesive compositions are non-inhibitory to the growth of microorganisms. Typically, the pH of the adhesive should be maintained at a pH of about 5 to 9, more preferably at a pH of about 6 to 8.

In a preferred thin film culture device, the amount of sample used (or to be evaluated) is contained on the substrate by the components of the medium alone. In an
30 alternate embodiment, a device may include a sample-containing foam layer. A foam spacer, not shown, having a circular opening in the foam is adhered to the medium

coated surface of substrate 12. The foam spacer which covers the periphery of substrate 12 defines the area which is to be inoculated with a sample and serves to prevent the sample from leaking from the substrate. Suitable materials for the spacer member are any solid non-inhibitory natural or synthetic substance which is readily available in sheet form but is not a microorganism growth site. Polyethylene, polypropylene, polyethylene terephthalate and polystyrene are a few examples of suitable synthetic materials. In particular, relatively inexpensive commercially available polystyrene foams and polyethylene foams are preferred, and polystyrene foam is presently most preferred. Natural substances such as cellulose sheets, metal e.g. foil sheets, wood and the like are suitable alternatives.

A cover sheet 20 is attached to one edge of an upper surface of substrate 12. Cover sheet 20 is preferably made of a transparent film or sheet material in order to facilitate counting of bacterial colonies present on the substrate. In addition, cover sheet 20 is preferably impermeable to bacteria and water vapor in order to avoid the risk of contamination and deterioration of the components. A preferred material for use as a cover sheet 20 is biaxially-oriented polypropylene.

In a manner similar to coating adhesive and medium particles on a surface of substrate 12, a surface of cover sheet 20 is also coated with adhesive and reconstitutable culture medium particles. In a preferred embodiment, the layer of adhesive 22 includes an indicator dye to aid detection and enumeration of growing bacterial colonies.

Generally, cover sheet 20 will have the same properties, such as transparency and preferred water impermeability, as substrate 12. Furthermore, cover sheet 20 may have imprinted patterns or a mask-edge (not shown) to aid in the counting of microorganism colonies, to provide a target for placement of the aqueous test sample, and/or for aesthetic reasons.

Cover sheet 20 may be selected to provide the amount of oxygen transmission necessary for the type of microorganism desired to be grown. For example, polyester films have a low oxygen permeability (less than $0.78 \text{ g}/100 \text{ cm}^2/24 \text{ hours}$ per 25μ of thickness), and would be suitable for growing anaerobic bacteria. On the other hand, some forms of polyethylene have a relatively high oxygen permeability (approximately $78 \text{ g}/100 \text{ cm}^2/24 \text{ hours}$ per 25μ of

thickness), and would be suitable for the growth of aerobic organisms, with or without the use of an air permeable membrane. One preferred material for cover sheet 20 is a 1.6 mil biaxially-oriented polypropylene film. Another preferred material for the cover sheet is a commercially available polyethylene terephthalate treated with an antifog agent (commercially available as FSI-47 from Film Specialties Inc., Whitehouse, NJ). Another preferred material is sol-gel treated polyethylene terephthalate (commercially available as SCOTCHPAR Brand film No. FE 40492 from 3M, St. Paul, MN). The cover sheet 20 may be affixed by conventional methods such as heat sealing, adhesives, double coated adhesive tapes and the like.

Suitable indicator dyes for use in the present invention include compounds which are metabolized by the growing organisms and which become colored due to the action of the metabolites produced by developing bacterial colonies. The visual change in color allows for easier detection and visualization of the growing colonies. Preferred indicator dyes include reduction sensitive dyes such as triphenyltetrazolium chloride, p-tolyl tetrazolium red, tetrazolium violet, veratryl tetrazolium blue and related dyes. Other suitable dyes include dyes which are sensitive to pH changes such as neutral red.

Triphenyltetrazolium chloride is a preferred dye for use in devices designed to culture bacteria which may be found in food products such as *S. aureus*, *Micrococcus*, or other types of bacteria which may be commonly found in food products such as milk or other dairy products.

In use, a predetermined amount of inoculum, typically about one milliliter of inoculum, is added to the device illustrated in Fig. 1 by pulling back cover sheet 20 and adding an aqueous test sample or water to the middle of substrate 12. Cover sheet 20 is then replaced over substrate 12 and the inoculum is evenly spread on the substrate. A convenient tool to do this spreading is a weighted circular template which also is used to confine the inoculum to a specific area of substrate 12. As the inoculum contacts and is spread on substrate 12, the culture medium on substrate 12 hydrates to form a growth-supporting nutrient gel. The inoculated device is then incubated for a

predetermined time after which the number of bacterial colonies growing on the substrate may be counted through the transparent cover sheet 20.

Although the use of the medium particles of this invention on a thin film device is described above, those of ordinary skill in the art will recognize that the medium
5 particles may be used in other culturing devices which are known in the art.

The following examples are intended to provide further details and embodiments related to the practice of the present invention. These examples are provided for illustrative purposes and should not be construed to limit the scope of the present invention which is defined in the appended claims.

10

EXAMPLES

EXAMPLE 1

Three pre-weighed powders, 12.7 kilograms of xanthan gum (KTLTF, available from Kelco. Co.), 12.7 kilograms of locust bean gum (GENU, available
15 from Hercules) and 6.4 kilograms of re-extracted guar gum (available from Rhone-Poulenc) were placed into the loading bowl of a 60 kilogram capacity Glatt fluid bed agglomerator to produce medium particles. The fluid bed agglomerator was fitted with silicone gaskets and a filter sock with a 2 micron nominal pore size. The fluid bed agglomerator was cycled for 30 seconds to facilitate mixing of these
20 powders and then 8.0 kilograms of standard methods nutrients, including sugars, proteins, and salts (available from Acumedia Inc.) was added to the loading bowl. Again, the fluid bed agglomerator was cycled for another 30 seconds of mixing.

3.5 liters of a dilute phosphate buffer binding spray solution made according to the Standard Methods, containing potassium phosphate and magnesium chloride,
25 were loaded to the spray pump of the fluid bed agglomerator. The machine blower, heater, and spray pump were started at the same time.

The binding spray solution was added at the rate of 10 milliliters per 3 minutes per kilogram of powder, through a top-mounted spray port of 1.5 millimeters in diameter. All the binding spray solution was added in the first
30 25-30 minutes of the 55-65 minute total cycle time.

The specifications of the resulting medium particles included at least 70% of the particles in the range of 325 to 140 mesh which had less than 5 wt.% moisture content.

- 5 The following table shows the temperature, percent moisture, and density (grams per cubic centimeter) of the product, the amount of diluent added, and the elapsed time.

Temp. of Product (°C)	Total Diluent (ml)	Time (min.)	Moisture	Density
23				
23	400	5		
22	750	10		
24	1100	15		
27	1500	18		
28	2500	20		
28	2500	23		
29	3000	26		
30	3500	29		
31		35		
33		45		
34		55	8.3	.51
35		60		
36		65	5.1	.57

EXAMPLE 2

- 10 A silicone coated paper web approximately 4-5 mils thick with a grid pattern printed on the side opposite the silicone coating (available from Schoeller Technical Papers) was coated at a coating weight of about 0.15 grams per 24 square inches (dry coating weight) with a solution of isooctyl acrylate/acrylamide adhesive and then dried. The adhesive was coated on the side with the preprinted grid; that is, on
- 15 the side opposite the side coated with the silicone.

- Medium particles were made by the fluid bed agglomeration process of Example 1 and these medium particles were coated onto the adhesive layer by passing the paper web through a cloud of suspended medium particles. The medium particles which contacted the adhesive adhered to the coated paper web,
- 20 and the excess medium particles were shaken off the web. The coating weight of

media particles was approximately 0.4 grams per 24 square inches. This coating was substantially water-free and cold-water reconstitutable.

5 A second web of transparent polypropylene was coated with a solution of the isooctyl acrylate/acrylamide adhesive at a coating weight of about 0.15 grams per 24 square inches (dry coating weight). This adhesive contained the indicator dye triphenyl tetrazolium chloride. Medium particles were coated at approximately 0.4 grams per 24 square inches onto the adhesive layer of the polypropylene web in the same manner as the medium particles were coated onto the silicone coated paper. The polypropylene was substantially bacteria and water vapor impermeable.

10 The two webs were brought together, with the sides coated with medium particles facing each other. A rectangle 3 inches by 4 inches was cut out from the combined webs, and the webs were then heat-sealed together along one edge in a hinge-like fashion.

15 EXAMPLE 3

A stock phosphate buffer solution was prepared by dissolving 34 grams of monobasic potassium phosphate in approximately 500 milliliters of microbiologically suitable (MS) water in a one liter volumetric flask. The pH was adjusted to 7.2 with 1N sodium hydroxide, and MS water added to bring to volume.

-20 The stock buffer was sterilized by autoclaving at 121°C for 15 minutes. Storage was at 0°C. Working phosphate buffer (hereinafter referred to simply as phosphate buffer) was prepared by adding 1.25 milliliters stock buffer to MS water in a one liter volumetric flask and diluting to the mark. The phosphate buffer was autoclaved at 121°C for 15 minutes.

25 Eleven grams of fresh, raw milk were added to 99 milliliters phosphate buffer solution to prepare the initial 1:10 dilution. Samples were mixed by shaking the diluted samples 25 times in an arc of approximately 30 centimeters in 7 seconds. Diluents are preferably at about the same temperature as the sample in order to avoid additional stress to bacteria that are present.

From the initial 1:10 dilutions, serial 10-fold dilutions were made by transferring 11 milliliters of well-mixed, diluted sample to 99 milliliters diluent. Samples of dilutions of 10^{-2} and 10^{-3} were used.

Standard methods agar (available from BBL Co.) was prepared according to the manufacturer's direction. The standard methods agar was autoclaved for 15 minutes at 121°C . The agar was then placed in a water bath to allow the agar to reach a temperature of 45°C in order to melt and temper the agar. One milliliter of the appropriately diluted sample was pipetted into each of the petri dishes. 10 to 12 milliliters of melted, tempered agar was then poured into each petri dish. As each dish was poured, the dish was swirled gently to mix the sample and agar and to distribute the mixture evenly across the plate. The agar was allowed to solidify. The plate was then inverted and incubated for 45-48 hours at 32°C .

Standard aerobic count PETRIFILM plates (catalog No. 6400, 3M, St. Paul, MN.) as well as the plates made in Example 2 were also used to test appropriately diluted samples. For both of these plates, the top clear film of the plate was lifted and one milliliter of diluted sample was pipetted onto the center of the bottom film. The top film was allowed to drop onto the inoculum. A plastic spreader was placed, recessed side down, over the center of the sample. The sample was dispersed by pressing on the center of the spreader. The gel was allowed to solidify, and the plates were then inverted and incubated for 45-48 hours at 32°C .

The colonies were counted after the incubation time with a Quebec colony counter. Counts ranging from 15 to 300 were used to calculate the mean raw counts for each milk-medium combination except when the plate had counts of 15 or less (samples 14 and 30, Table 1). One sample, number 9, was "too numerous to count" on all plates, and so was discarded.

Results from 30 fresh, raw milk samples are shown in the following table. These counts are averages of two duplicates.

Sample	Log of Dil.	Agar	Control	Lot 001	Lot 002
1	3	202	170	109	115
2	2	53.5	96	109.5	90.5
3	2	12	23	24.5	31
4	2	60	59	63	59
5	3	102	144	141.5	136
6	2	55.5	64.5	74.5	68.5
7	2	49.5	79	83	67
8	2	30.5	31.5	39.5	28.5
9	tntc	tntc	tntc	tntc	tntc
10	2	23	22.5	26.5	28.5
11	3	71.5	102	181.5	75.5
12	3	132	169.5	144.5	155.5
13	2	45	74.5	71	72
14	2	8	12	10	11.5
15	3	105	216.5	209	188.5
16	2	48.5	67.5	57.5	62
17	3	29.5	44.5	43.5	37
18	2	47	58	84	99.5
19	2	25	29.5	23.5	32
20	2	21	19.5	31.5	22.5
21	2	262.25	213.7	290.3	255.7
22	2	14.5	13.5	20.5	23.5
23	2	82	122.5	105.5	105
24	2	34.5	42.5	60	57.5
25	2	15	18	24.5	28.5
26	2	85.5	85	72.5	70.5
27	3	68	93.5	89	69.5
28	3	105.5	145.5	118	134.5
29	3	137.5	133.5	114.5	122
30	2	8.5	6.5	12	6.5

CLAIMS

1. A reconstitutable culture medium comprising
 - 1) nutrients for growing microorganisms, and
 - 2) a mixture of gelling agents,wherein the nutrients and gelling agents are prepared in granular form by agglomerating the nutrients and mixture of gelling agents in the presence of an aqueous binder in a fluidized bed.
2. The reconstitutable culture medium of claim 1 wherein the nutrients are selected from the group consisting of proteins, carbohydrates, salts and mixtures thereof.
3. The reconstitutable culture medium of claim 1 wherein the gelling agents are gums.
4. The reconstitutable culture medium of claim 1 comprising about 15-25 wt.% nutrients and about 75-85 wt.% gelling agents.
5. A method of making medium particles comprising the step of agglomerating nutrients for growing microorganisms and a mixture of gelling agents in the presence of an aqueous binder in a fluidized bed.
6. The method of making the medium particles of claim 5 wherein the nutrients are selected from the group consisting of proteins, carbohydrates, salts and mixtures thereof.
7. The method of making the medium particles of claim 5 wherein the gelling agents are gums.

8. The method of making the medium particles of claim 5 wherein a mixture of the nutrients and gelling agents contacts a sufficient amount of an aqueous binder to form particles having a diameter greater than about 40 microns and then drying the particles at a temperature less than about 40°C until the particles have a moisture content of less than about 5 wt.%.
5

9. The method of making the medium particles of claim 5 further comprising the step of collecting particles having a diameter in the range of about 50-150 microns and a density in the range of about 0.5-0.6 g/cm³.
10

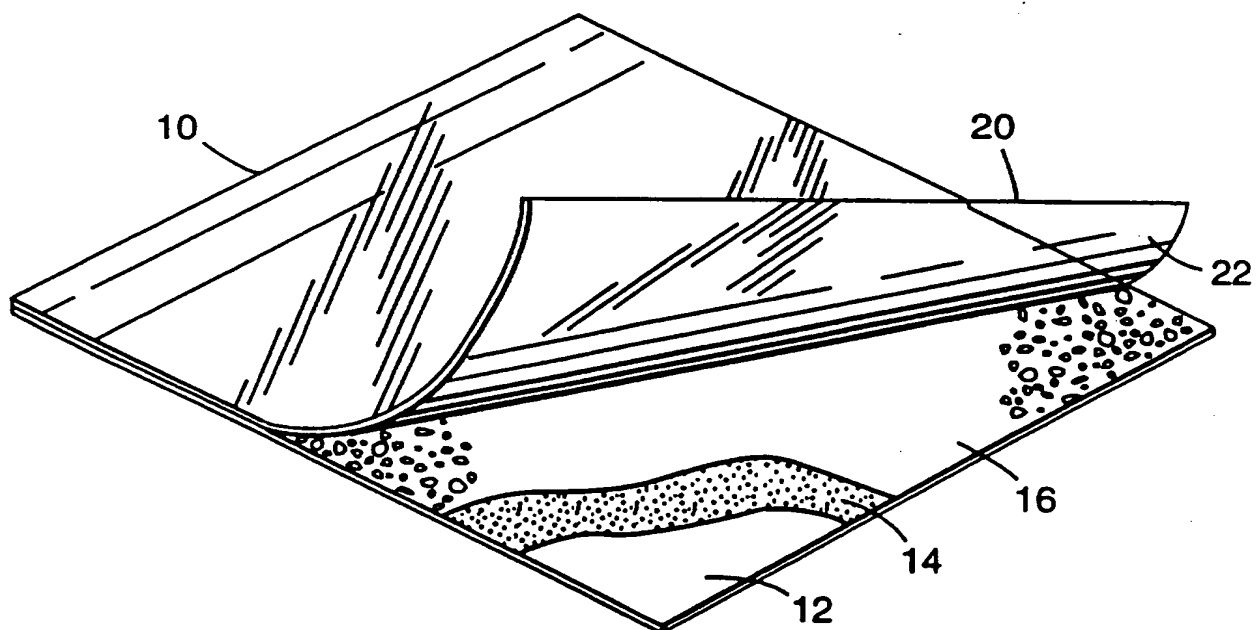
10. A thin film culture plate device for growing microorganisms comprising

1) a self-supporting, waterproof substrate containing a layer of a reconstitutable culture medium of claim 1 and

2) a cover sheet adhered to a portion of the substrate.

15

1/1

**Fig. 1**

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/14536A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N1/00 C12N1/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 755 468 A (JUNG GERARD ET AL) 5 July 1988	1-3,5-9
Y	see column 5, line 25 - line 35; claims 1,4,5,9,11,13,14; example 1	1-10
Y	EP 0 398 703 A (MINNESOTA MINING AND MANUFACTURING COMPANY) 22 November 1990 cited in the application see example 1	1-10
Y	US 4 565 783 A (HANSEN PAUL E ET AL) 9 October 1984 cited in the application see claims; examples	1,10
A	US 3 202 731 A (A GREVENSTUK ET AL.) 24 August 1965 see claims; figures; examples	1,2,5,6
	-/-	

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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International Application No.

PCT/US 96/14536

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